University of North Carolina Asheville
Journal of Undergraduate Research
Asheville, North Carolina
May 2018

# Cloning of Vomeronasal Type-2 Receptors for Deorphanization

Ariane Eceiza
Department of Biology
The University of North Carolina Asheville
One University Heights
Asheville, North Carolina 28804 USA

Faculty Advisor: Dr. Angel Kaur

## Abstract

The vomeronasal organ (VNO) is a chemosensory organ present in amphibians, reptiles, and non-primate mammals. In mice, vomeronasal neurons express vomeronasal-1 receptors (V1R) or vomeronasal-2 receptors (V2R), both of which are G protein-coupled receptors involved in pheromone detection. V2Rs are expressed by the basal neurons of the VNO. They differ from V1Rs in their sequence length and G-protein linkage, and are of special interest because they are used to detect proteins, major urinary proteins (MUPs), which induce intermale aggression, female responsiveness to mating, and territory marking. Because V2Rs use combinatorial coding instead of a labelled line, linking pheromone responses to the correct V2R has so far been difficult; even though each cell expresses a different V2R, each V2R could respond to multiple MUPs and each MUP could activate multiple V2Rs. This paper describes a cell culture method that would allow for deorphanizing of V2Rs by creating entire cell populations which only express a single V2R. With V2Rs deorphanized, mapping of pathways can begin from a bottom-up method, instead of the more difficult top-down approach. This paper shows how to isolate and clone V2Rs for eventual individual expression in mammalian cells using DNA purification, Zero Blunt TOPO cloning and ligation into mammalian vector. V2Rs 83 and 121 were cloned into a bacterial vector. Currently, work is being done to understand if the blunt end cloning did result in capturing of a complete V2R sequence in the correct orientation for future transfer to mammalian vectors. These results demonstrate progress towards setting up a cell culture based V2Rs expression system. This system will allow for further research on the identification of receptor-ligand interactions, enabling a reliable study of how external environmental cues can direct behaviors.

## 1. Introduction

The vomeronasal organ (VNO) is a chemosensory organ of the accessory olfactory system present in amphibians, reptiles, and non-primate mammals. The VNO is specialized in detecting non-volatile chemical cues[5] that transmit chemical information between individuals of the same species, known as pheromones. These chemical cues are detected by receptors that send signals first to the olfactory bulb, then continue to the amygdaloid nucleus[10], and are transformed by the hypothalamus to responses such as mating, male aggression and fear behaviors[7]. Research has shown that the removal of the vomeronasal organ can cause dramatic effects on animal sexual behaviors by decreasing both the female and male responsiveness to mating[8,9].

The VNO detects pheromones through the G-coupled protein Vomeronasal Type 1 Receptor (V1R), located on the luminal side of apical neurons, and Vomeronasal Type 2 receptor (V2R), located on the basal neurons. These two receptors differ in their sequence length and G-protein linkage. It is believed that the presence of two different receptors on the VNO is due to their differences on the detecting stimuli pathway and the ligands that they recognize[4]. The V2 receptors are part of the C family of the GPCRs, including the metabotropic glutamate receptors (mGluR), extracellular calcium sensing receptors (CaSR), and GABA-B receptors[1]. These receptors are of special interest because they are used to detect protein pheromones, the Major Urinary Proteins (MUPs), which induce intermale aggression, female responsiveness to mating, and territory marking behaviors[2], and can detect MHC peptides[3] as well.

Research conducted on V2Rs present many challenges due to the lack of specificity in the pheromone-receptor interaction. Most pheromones follow the "labelled line" coding model, in which a pheromone activates a dedicated receptor, which in turn triggers a specific neural circuit resulting in a defined innate behavior. In contrast, researchers found that MUPs follow a combinatorial coding model in which the MUP-receptor interaction does not follow the one ligand – one receptor pattern[6]. Instead, each MUP can activate multiple receptors and each receptor can be activated by multiple MUPs. The ability of pheromones to activate a broad range of V2Rs produces an increased reception capacity and thus the coding ability for MUPs in the mouse without having to increase the number of available MUP ligands. This complex reception system adds a layer of difficulty in the study of the pheromone-receptor interaction and further study of the neural activation and development of behavior.

Researchers such as Dey and Matsunami (2011) have previously studied the deorphanization of V2Rs but did not obtain conclusive results due to the many challenges of the project. The expression of V2Rs on the ER lumen limited the study of their ligand interaction. However, some steps towards the successful completion of the project were made by studying the connection between knocking down the chaperone calreticulin in a HEK293T derived cell line and V2R export to the cell membrane[3]. The goal of this project is to successfully clone the 130 V2 receptors by using a C57BL/6j VNO cDNA library, DNA purification, Zero Blunt TOPO cloning and ligation to mammalian vector. The cloning of the V2Rs for deorphanization and the export to the cellular surface would allow for an easier study of pheromone-receptor interaction and future research on the complexity of the stimuli and activation pathway necessary to understand the mechanism that drives behavioral response.

## 2. Methods

### 2.1 DNA Amplification of V2Rs from VNO cDNA

Primers targeting full length V2R sequences (V2R 121: AGGTGTAACTGTGTGTGTGATGT, V2R 83: CACTCAATCAAGGCGCTTCAC) were designed using the sequence information available in ensemble.org. V2R sequences were amplified from VNO cDNA libraries using a Phusion polymerase system. GAPDH primers were designed and used for control reactions. Reactions were immediately transferred to a preheated thermocycler to 98°C, and run on the following program: 1 cycle 98°C for 3 minutes, 34 cycles (98°C for 10 seconds, 61°C for 30 seconds, 72°C for 2 minutes), and 1 cycle 72°C for 10 minutes.

Amplified DNA fragments were visualized on a 1.2% Agarose gel containing 0.5$\mu$g/mL ethidium bromide in 1X TBE, using a 1kp ladder as reference. Sequences of the expected length (2500-3000bp) were excised from the gel and purified using PureLink Quick Gel Extraction and PCR Purification Combo Kit.

### 2.2 PCR Product Cloning

Zero Blunt TOPO cloning PCR kit was used to clone the purified DNA. The reaction was incubated at room temperature for 5 minutes. 2$\mu$L of the reaction were added to competent cells and incubated on ice for 30 minutes. Competent cells were heatshocked at 42°C for 30 seconds and immediately transferred to ice for 2 minutes. 250$\mu$L of S.O.C. were added to the sample and incubated at 225 rpm for 1 hour. Transformed bacteria were plated on Ampicillin selective plates and incubated overnight at 37°C. 10 colonies were grown overnight and plasmid DNA was purified via PureLink Quick Plasmid Miniprep Kit and PureLink Hipure Plasmid Maxiprep Kit.

### 2.3 Restriction Digest

Isolated plasmids were subjected to restriction digest with HindIII or HindIII and EcoRI co-digest for 1 hour at 37°C to confirm successful cloning. Restriction digest fragments were visualized on a 1.2% Agarose gel containing 0.5$\mu$g/mL ethidium bromide in 1X TBE, using a 1 kb ladder as reference.

## 2.4: Sequence Analysis via PCR

Successful cloning reactions were further confirmed by use of sequential forward primers, which targeted 500-800bp sections of the V2R sequence. Plasmid DNA amplicons were compared to original VNO cDNA amplicons using the same primers.

Reactions were immediately transferred to a preheated thermocycler to 98°C, and were run on the following program: 1 cycle 95°C for 5 minutes, 34 cycles (95°C for 30 seconds, 55-60°C for 30 seconds, 72°C for 1 minute), and 1 cycle at 72°C for 10 minutes.

Sequential primer fragments were visualized on a 1% Agarose gel containing 0.5$\mu$g/mL ethidium bromide in 1X TBE, using a 1 kb ladder as reference.

# 3. Results

## 3.1 Gel Visualization and Extraction

V2R 83 and 121 were amplified using previously designated directed primers and were visualized via gel electrophoresis. Successful PCR amplification resulted in bands visualized between 2500bp and 3000bp, which were extracted for DNA purification and TOPO cloning reaction.

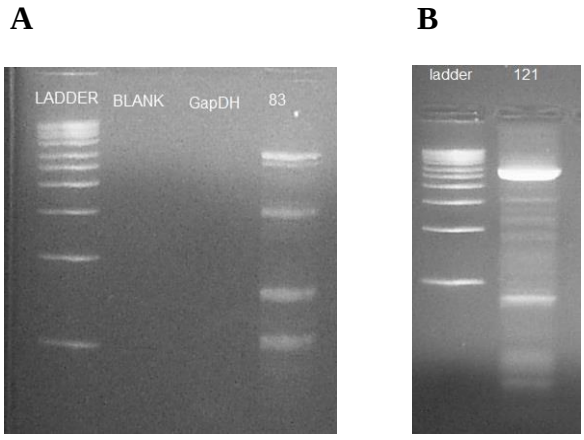


Figure 1. V2R 83 and 121 following amplification

Figure 1. V2R 83 and 121 amplifications through gel electrophoresis for purification. (a) V2R 83 and positive control GAPDH amplification from a VNO cDNA library using directed primers designed to amplify the full coding sequence. (b) V2R 121 amplification from a VNO cDNA library using directed primers designated to amplify the full coding sequence. The bands located between the 2500bp and 3000bp were extracted for DNA purification.

## 3.2 Restriction digest

After Zero Blunt TOPO cloning was performed on the purified DNA, double restriction digest of HindIII and EcoRI and control digest of HindIII were carried out to assess the presence of the cloned sequence and linearize and separate sequences. Successful results presented one band for the control single digest of HindIII and three bands for the double digest of HindIII and EcoRI. Successful results were obtained for V2R 121 (Fig. 2a), while results for V2R 83 were inconclusive (Fig. 2b, 2c).

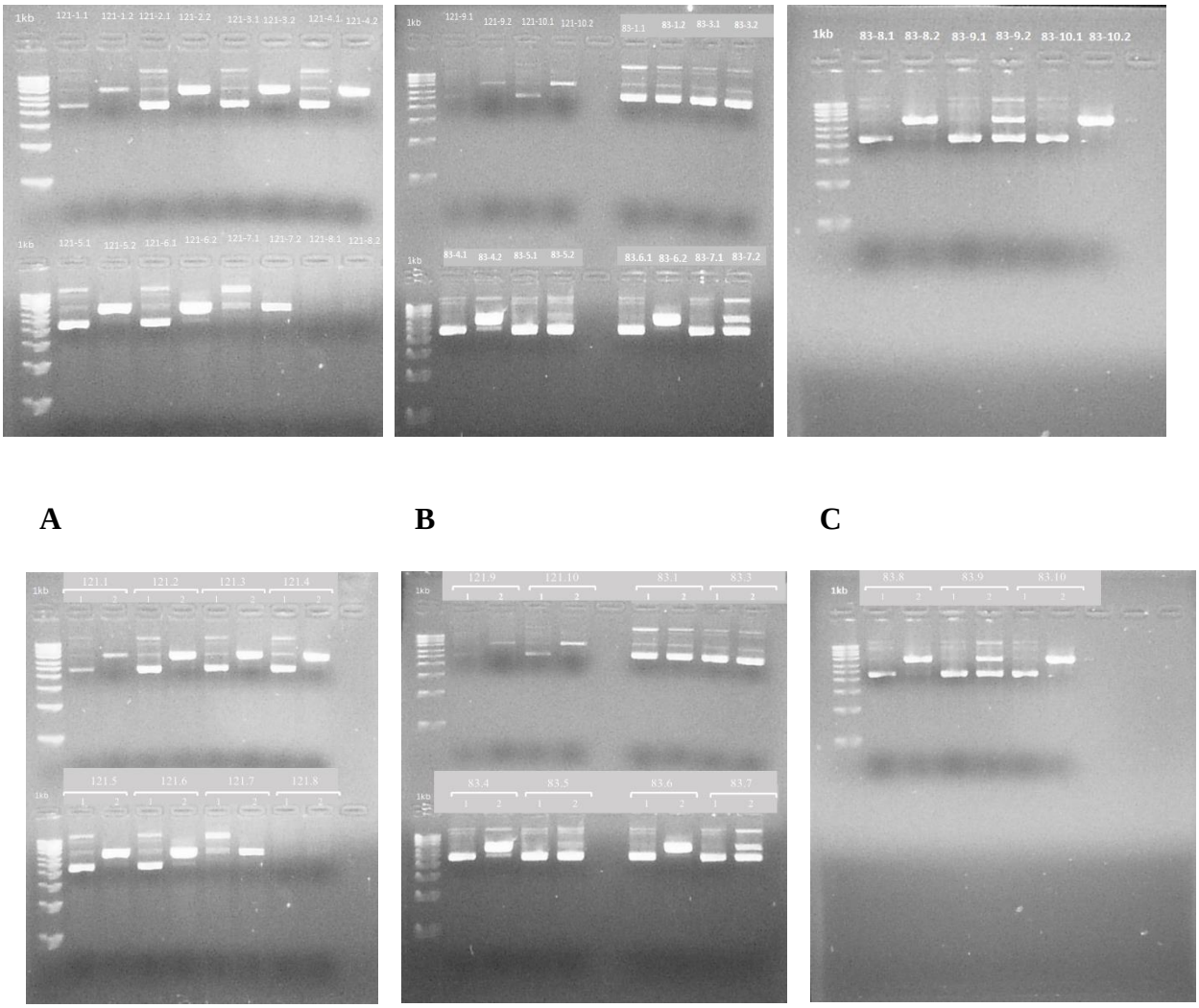


Figure 2. V2R 121 and 83 following restriction digest

Figure 2. Restriction digest of HindIII and EcoRI of V2R 121 and 83. Successful results showed three bands for the double digest of HindIII and EcoRI and one band for single digest of HindIII. (a); V2R 121 1.1-8.2 restriction digest. (b); V2R 121 9.1-10.2 and V2R 83 1.1-7.2 restriction digest. (c); V2R 83 8.1-10.2 restriction digest.

## 3.3 Sequence analysis

Sequential analysis of cloned sequences was carried out to determine if the correct and complete sequence had been cloned. 5 sequential primers were designed to amplify the specific V2R sequence in 600 to 800 bp sections. Plasmids V2R 121.2 and 121.3 appear to contain full length sequences (Fig. 3a, 3b). V2R 83.7 and 83.9 were considered successful results although they presented light bands around 600 to 800bp due to the use of only forward primers during the PCR reaction (Fig. 3e).

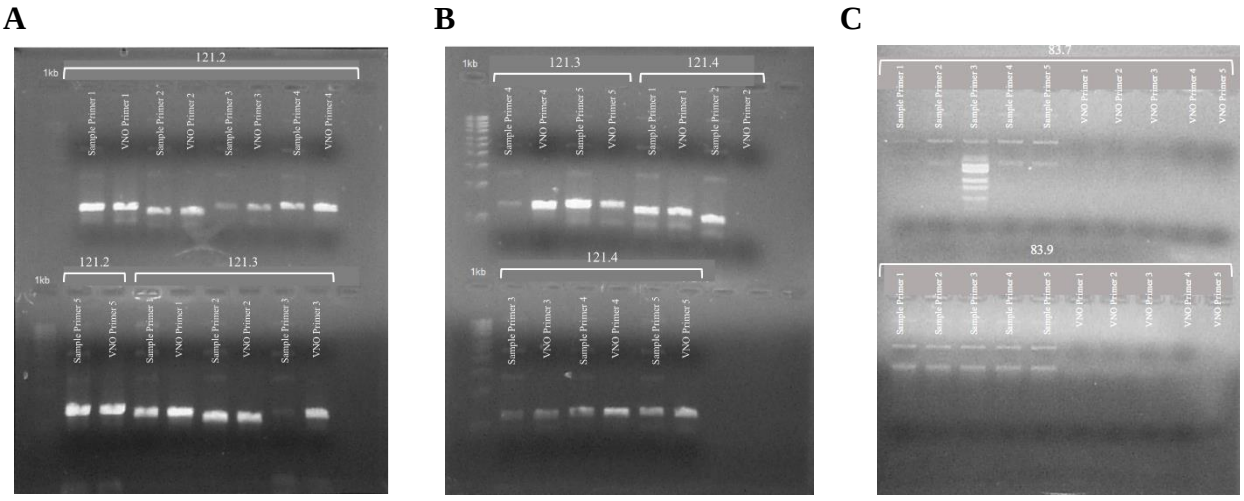


Figure 3. V2R 121 and 83 following sequential analysis

Figure 3. Sequential analysis of V2R 121 and 83 to assay for presence of full-length clones using 5 sequential primers and VNO cDNA as positive control (a); V2R 121 2.1-3.3 sequence analysis. (b); V2R 121 3.4-4.5 sequence analysis. (c); V2R 83 7.1-9.5 sequence analysis.

## 4. Discussion

To date, V2Rs 121 and 83 have been successfully amplified, purified and cloned. In-house sequencing diagnostic results suggest a presence of a complete sequence in V2R plasmid clones 121.1, 121.2, 121.3, 121.4, 121.6, 83.7, and 83.9. Sanger sequencing results from Genewiz reported no priming. This could be due to the presence of TE buffer in the sequence primers containing a high concentration of EDTA, which could produce an error in the sequencing reaction. The next step of the project would be to re-send the samples for sequencing. Possible errors in the procedure could lead to errors such as the insertion of incorrect sequences on the plasmid, mutation on the sequence or insertion of the sequence in the wrong orientation. However, it is expected that V2R 121 would be able to be cloned successfully due to the close sequence similarity to receptor 122, which has been previously cloned following the same procedure, as well as multiple previous attempts that suggested a high efficacy on the cloning method for this particular receptor. Upon successful results, the samples would undergo phosphatase treatment to assure correct orientation on the insertion to a mammalian vector. The next step of the project would be to transfect the mammalian vector into mammalian cells to induce expression of the cloned V2R on the cell surface for further study.

Little research has been published regarding the deorphanization of V2Rs. This project proposes a possible mechanism for receptor cloning that could be used to generate a vomeronasal receptor library. The expression of the receptors on the cellular surface would facilitate the study of the combinatorial coding model used in the MUPs-V2Rs interactions. By successfully cloning the receptors, the study of the behavioral response towards external stimuli would be possible, permitting the study of neural development and pathways of behaviors such as anger and mating response.

## 5. Acknowledgements

The author wishes to express her appreciation to her research advisor Dr. Angel Kaur, the UNC Asheville Neuroscience program and the UNC Asheville Biology program. She would also like to thank her partners on this project: Walter Evan Brown, Caiti Lahue, Zachary Kerkenbush, and the other members of the Kaur research lab for their help and support.

## 6. References


1. Alioto T. S., Ngai, J. 2006. The repertoire of olfactory C family G protein-coupled receptors in zebrafish: candidate chemosensory receptors for amino acids. *BMC Genomics* 7:309.

2. Chamero, P. *et. al.* 2007. The Genetic Basis of Individual-Recognition Signals in the Mouse. *Current Biololgy* 17:1771-1777.

3. Dey S., Matsunami, H. 2011. Calreticulin Chaperones Regulate Functional Expression of Vomeronasal Type 2 Pheromone Receptors. *PNAS* 108, 40:16651-6656.

4. Døving K. B., Trotier, D. 1998. Structure and function of the Vomeronasal organ. *The journal of Experimental Biology* 201:2913-2925.

5. Kannan S., Bharathiraja, M. K. 2008. Regulatory Mechanism of Mouse Sex Pheromone and Intracellular Calcium Level in V2Rx Receptor Expressing Cells. *Current Science* 95, 3:397-400.

6. Kaur, A. *et. al.* 2014. Murine Pheromone Proteins Constitute a Context-Dependent Combinatorial Code Governing Multiple Social Behaviors. *Cell* 157, 3:676-688.

7. Keverne, E. B. 1999. The Vomeronasal Organ. *Science* 286, 5440:716-720.

8. Powers J. B., Winans, S. 1975. Vomeronasal organ: Critical role in mediating sexual behavior of the male hamster. *Science* 187, 4180:961-963.

9. Winans S., Power, J. B. 1977. Olfactory and Vomeronasal deafferentation of male hamster: histological and behavioral analyses. *Brain Res* 126:325-344.

10. Winans S., Scalia, F. 1970. Amygdaloid Nucleus: New Afferent Input from the Vomeronasal Organ. *Science* 170, 3955:330-332.